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Quantification and Molecular Characterization of *hprt* Mutants of Human T-Lymphocytes

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Somatic mutations have been implicated as critical early events in carcinogenesis. Point mutations, **deletions, and translocation events have been shown to activate oncogenes or inactivate suppressor oncogenes. In human population monitoring, quantitative analysis of mutation events that affect gene function is limited to those genes whose cellular phenotypes can be identified by selection procedures and to those tissues (like blood) that are accessible for analysis. In an effort to determine the frequency and types of mutations that can be detected at the hypoxanthine guanine phosphoribosyltransferase (*hprt*) gene, we have used the T-cell cloning assay and have developed a strategy to propagate mutants and screen for point mutations and breakage events. Early in the clonal expansion of mutants, 1–2 $\times$ 10^4 cells are prepared as a crude cell lysate, and a sample is analyzed using the multiplex polymerase chain reaction (PCR). Those mutants that yield altered DNA fragments are then expanded for Southern blot hybridization, PCR, flanking probe isolation, and DNA sequencing. To date we have found presumed point mutations, intragenic deletions, and deletions that extend outside of the *hprt* gene. By analyzing mutations in selectable, nonessential gene markers, it should be possible to understand mechanisms of both spontaneous and induced genetic damage. An association of these specific genetic events with human diseases and the evaluation of the ability of environmental chemicals to induce these specific types of mutations will lead to a rational basis for evaluating risks from various chemical exposures.**

Introduction

When evaluating the risks to human populations from particular environmental exposures, it is important to approach the analysis with an integrated investigation. The most informative approach is to combine environmental exposure monitoring with biological techniques that can provide insight into actual internal human exposure, dose to a relevant target tissue, markers of biological effect and, ultimately, measures of human disease. Although the most useful data on which to conduct a risk assessment may come from epidemiological studies identifying cancer or other human disease, one would like to identify potential adverse exposures before significant numbers of individuals are affected. Thus, it seems reasonable to develop and characterize markers of biological effect for identifying adverse exposure scenarios.

Because of the association of mutational events with the etiology of cancer, a number of genetic damage assays have been used to detect the potential of various chemicals and physical agents to cause genotoxic effects. The range and types of mutational events associated with cancer and other diseases include point mutations, deletions, translocations, mitotic recombination, gene conversion, gene amplification, and aneuploidy. It seems obvious that an analysis of various genetic assays should include an understanding of the spectrum of genetic damage detected by that assay.

In human population monitoring, the quantitative analysis of mutation events that affect gene function is limited to those genes whose cellular phenotypes can be identified by selection procedures and to those tissues (like blood) that are accessible for analysis. To determine the frequency and types of mutations that can be detected at the *hprt* gene, we have used the T-cell cloning assay (*1,2*) and developed a strategy to propagate mutants and screen for point mutations and breakage events.

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Earlier, *in vitro* studies using quantitative comparisons between autosomal markers (like thymidine kinase [tk]) and X-linked markers (like *hprt*) have demonstrated that autosomal markers can detect the clastogenicity of chemicals, whereas X-linked markers severely underestimate the clastogenicity of test chemicals (*3*). One possible mechanism for these differences is the potential nonviability of *hprt* mutants containing multilocus deletions. Thus, to understand the ability of the *hprt* locus of lymphocytes to detect large-scale deletions, we have initially concentrated on the analysis of *hprt* deletion mutants.

Materials and Methods

Healthy volunteers were enrolled by advertisements in the local media. Informed consent was obtained using procedures approved by Institutional Review Boards for the protection of human subjects. Specimens of anticoagulated peripheral blood were obtained and processed on the same day. Mononuclear cells were isolated by density gradient centrifugation.

Cryopreservation of Lymphocytes

Lymphocytes were counted using a hemocytometer, centrifuged, and resuspended in 20% defined/supplemented bovine calf serum (CBS; Hyclone, Logan, UT; heat inactivated for 30 min at 57°C), 70% RPMI 1640 medium (RPMI; Gibco, Grand Island, NY), and 10% dimethyl sulfoxide. Twenty million lymphocytes were aliquoted into cryogenic vials. All vials were placed in a cryomed cell freezer (Cryomed; Cryomed, New Baltimore, MI) at an internal temperature of 4°C. The cryomed cooled the cells at 1°C/min until the temperature reached −45°C and then at 10°C/min until it reached −90°C. Cells were then stored in liquid nitrogen units.

Lymphocyte Culture

Frozen lymphocytes were rapidly thawed and placed in a mixture of 50% CBS and 50% RPMI. Cells were centrifuged at 250*g*, resuspended in 10 mL of RPMI, washed twice with RPMI, resuspended in 5 mL of RPMI, and viable cell number was determined using a hemocytometer to determine recovery and viability using eosin viability stain (Sigma, St. Louis, MO).

Cells for *in vivo* experiments were primed for 21–25 hr in 70% RPMI, 20% HL-1 medium (Ventrex, Portland, MN), 10% CBS, and 1 μg/mL phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC).

Feeder Cells

Two different feeder cell lines were used for these experiments. A spontaneous *hprt*-deficient subclone (provided by the University of Vermont) of the TK6 cell line (derived from the human lymphoblastoid cell line WIL-2) and the X3C cell line (*4*) (derived as an X-ray-induced *hprt* deletion mutant from a TK6 cell line, produced by Jean Whaley and provided by Howard Liber, Harvard School of Public Health). For use as feeders, cells were irradiated with 8000 rad from a cesium source.

In Vivo Assay

Cells were plated in 96-well, round-bottom, microtiter plates (Nunc, Naperville, IL) at cell densities of 1, 2, 5, and 10 cells/well to determine cloning efficiency and at 1–2 × 10^4 cells/well for *hprt* mutant selection. Cells were plated in 200 μL of medium containing 20% growth factor supplement, 20% HL1, 5% CBS, 55% RPMI, and 0.25 μg/mL PHA with 1 × 10^4 irradiated feeder cells/well. Growth factor supplement (kindly provided by the University of Vermont; Burlington, VT) is the cell-free supernatant medium used in the lymphokine activated killer-cell therapy. The method for its preparation is reported by Branda et al. (*5*). Selection plates were supplemented with 10 μM 6-thioguanine (6-TG, Sigma).

In Vitro Assay

For *in vitro* treatments, cryopreserved lymphocytes were thawed, washed with fresh medium, and counted, as above. Cells were then placed in tissue culture flasks and gassed with 5% CO_2 for overnight culture in a 37°C incubator. When cells in the G_0 phase of cell growth were desired for chemical treatment, cells were cultured in RPMI with 10% CBS. For chemical treatments using proliferating cells, 1 μg/mL of PHA was added to stimulate the cells into division. The cells were incubated for 21–25 hr, centrifuged, resuspended in RPMI, and counted with a hemocytometer. Cells were prepared at a density of 2 × 10^6 cells/mL in RPMI, and 5 mL of the cell culture were added to 50 mL centrifuge tubes for treatment. The test agent was added, cultures were gassed with 5% CO_2 in air, and placed horizontally in a 37°C incubator for 3 hr. After treatment, cells were centrifuged at 250*g* and washed three times with 10 mL of RPMI. After the final wash, cells were resuspended in 5 mL RPMI and placed into a 25-cm^2 tissue culture flask. The tube was rinsed with 5 mL of 40/10 medium (50% RPMI, 40% HL1, 10% CBS), and the rinse was added to the tissue culture flask. Cells were gassed with 5% CO_2 in air and placed in a 37°C CO_2 incubator for 2 days.

Two different techniques were used for the mutant expression-mutant selection phases of the experiment. The first technique [standard (*6*)], modeled after standard *in vitro* mutagenesis procedures, involved an expression period during which time the cell culture was maintained in suspension culture. After the expression period, the cells were plated in 96-well plates with the selective agent. The second technique (*in situ*), developed by O'Neill et al. (7), involved plating cells into the 96-well plates on day 2 after treatment, allowing the cells to grow and the newly induced mutants to be expressed in the 96-well plates. The selection agent was then added to the 96-well tray after the 5-day expression period. Before conducting the *in situ* protocol, the standard method was used to establish the appropriate concentration range for the chemical treatment.

Scoring

Viable clones were scored using an inverted phase contrast microscope. Selection plates were scored 10–12 days after cloning and were scored a second time 5–7 days later. Survival and cloning efficiency plates were scored 14–15 days after cloning. On those occasions when nonmutant clones were to be isolated for additional culture, these plates were first scored at 10–12 days, clones were isolated, and final scoring was completed at 14–15 days after cloning. Survival and cloning efficiency with and without selection were calculated using the Poisson distribution ($p_o = e^{-x}$, where p_o is the fraction of wells without colony growth). Survival and cloning efficiency with and without selection $= -lnP_o\ x$, where x is the average number of cells plated per well. The mutant frequency is the ratio of the cloning efficiencies in the presence and absence of 6TG selection.

Mutant Isolation

The *hprt* mutants were isolated from selected experiments. These cells were cultured for cryopreservation and molecular analysis. About one-half of the total volume of media, 0.10 mL, was removed and discarded from the top of each well containing a clone selected for isolation. The clone was then carefully resuspended using a 1-mL pipet and transferred to a single well in another 96-well microtiter plate. The well was filled with fresh growth media (20% growth factor supplement, 20% HL1, 5% CBS, and 55% RPMI with 0.25 μg/mL PHA) containing 10 μM 6TG and 0.25×10^6 irradiated feeder cells/mL. Subsequent refeeding and subculture were performed using the same medium (without 6TG) and feeder cells. When sufficient cells were present in the well, cells were resuspended in medium, and the culture was divided among 2 (or more) individual wells of the 96-well tray. At the two-well/mutant stage, one well was used for multiplex PCR analysis.

Using the strategy outlined in Figure 1, the multiplex PCR analysis was used to determine which mutants contained total or partial *hprt* gene deletions. Those mutants were cultured to the extent of their viability and prepared for cryopreservation and further molecular analyses. Mutants that did not appear to result from deletions were not cultured further.

Cryopreservation of Mutants

One-half of the media was carefully removed (and discarded) from the top of each mutant to be cryopreserved. Cells were resuspended and counted using a hemocytometer. If the cell density was less than 2×10^6 cells/mL, the cells were centrifuged for 10 min at 250*g* and resuspended in the supernatant at 2×10^6 cells/mL. A total of 0.9 mL of cells was added to a cryogenic vial, mixed gently with 0.9 mL of freezing media (84% CBS, 16% dimethyl sulfoxide), and the vial was placed in a freezing collar (Taylor-Wharton) for cryopreservation. The freezing collar was set in position E to allow the cells to cool at 1°C min to a temperature of −80°C and then placed in liquid nitrogen.

In some cases, mutants did not grow to a concentration that could be frozen in this manner. In those cases, cells were counted, centrifuged for 10 min at 250*g*, and resuspended in 1.8 mL of 90% CBS, 10% dimethyl sulfoxide containing a concentration of irradiated feeder cells required to bring the overall cell density (lymphocytes and feeder cells) to 3×10^6 cells/mL.

Multiplex PCR Analysis

Crude cellular extracts were prepared by a modification of the method described by Higuchi (*8*). Briefly, $1–2 \times 10^4$

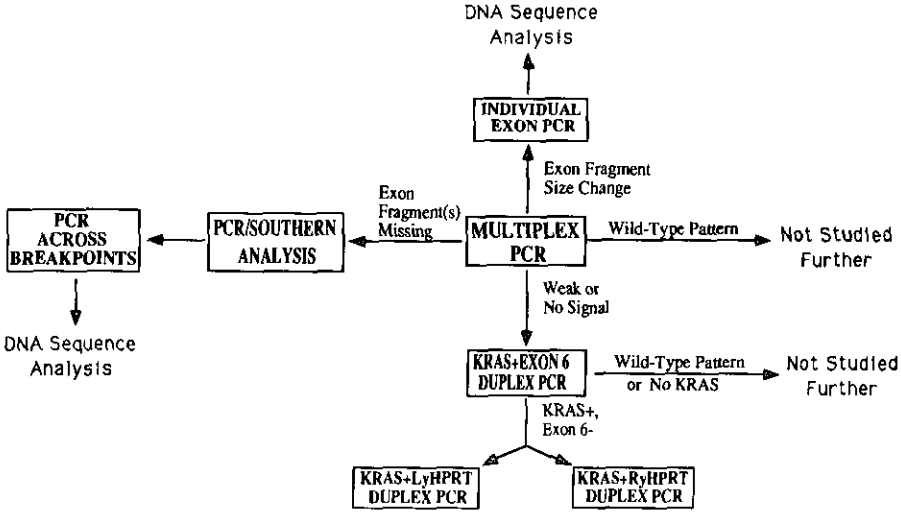

FIGURE 1. Schematic of strategy for molecular analysis of *hprt* deletion mutations. Rapid PCR-based screening of small samples of crude cell extracts are used to quickly identify *hprt* deletion mutants as described in the text.

cells were pelleted, washed in phosphate-buffered saline, and resuspended in 50 μL of lysis buffer consisting of 67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 6.8 μM EDTA, 0.45% Nonidet P40, 0.45% Tween 20, and 100 μg/mL proteinase K and incubated for at least 1 hr at 56°C. The proteinase K was then inactivated at 96°C for 10 min. The sample could be stored at −80°C for at least 6 months before use. Five to ten microliters, representing DNA from 1–4 × 10^3 cells, were used in a 50-μL multiplex *hprt* PCR reaction, described by Gibbs et al. (*9*). After 33 cycles, one-half of the reaction was analyzed by agarose gel (1.4%) electrophoresis for the presence of individual *hprt* exons and changes in exon fragment size. After the initial screening, larger amounts of crude extract at 2 × 10^6 cells/mL were prepared for further analyses. Direct DNA sequencing of PCR products was performed using primers and conditions described previously (*10,11*).

Strategy for Breakpoint Analysis

Figure 1 shows our strategy for identifying and analyzing *hprt* deletion mutations. Crude cell extracts were first analyzed by multiplex *hprt* PCR as described above. Those mutants that showed a wild-type pattern were not studied further. Those that showed a weak signal, or no signal, were candidates for containing total *hprt* gene deletions. The same result, however, would be obtained if the PCR reaction failed because of, for example, inhibitors from the extracts or inadequate DNA. These extracts were, therefore, examined in a duplex PCR reaction in which an unlinked gene segment (K-ras on chromosome 12) was coamplified with *hprt* exon 6 (*12*). If a wild-type pattern or no K-*ras* signal was seen after agarose gel electrophoresis, the mutant was not studied further. If K-*ras* was present, but the *hprt* exon 6 was absent, the mutation was a total *hprt* gene deletion. Flanking primer pairs located approximately 300 kb 3′ and 350 kb 5′ of the *hprt* gene were then used in a duplex PCR assay with the K-*ras* primers to determine how far the deletions extend beyond *hprt* (*12*).

If the original multiplex *hprt* PCR showed an alteration in an exon fragment size, that exon was individually amplified and sequenced (*11*) to determine the deletion size and the characteristics of the deletion junction. Hypotheses concerning mechanisms were then formed for testing.

Finally, when the multiplex *hprt* PCR revealed missing exon fragment(s), additional PCR reactions with intronic primer pairs and Southern blot hybridization were used to localize the breakpoints. The breakpoint junction was then amplified by PCR for DNA sequence analysis.

Results and Discussion

The mutant frequency data for 19 separate determinations is shown in Table 1. The mean mutant frequency for all samples was 9.5 × 10^{-6}, with a range of 2.1–27 × 10^{-6}. In addition to the mutant frequency, the age, sex, smoking status, nonselective cloning efficiency, number of cells plated for mutant selection, and the total number of mutants are presented. Although our database is small, it does appear that, as has been shown by other laboratories (*13,14*), there is an age-dependent increase in the mean mutant frequency (Fig. 2). There does not appear to be an increased *hprt* mutant frequency among smokers. To date, we have analyzed 165 mutants from 5 individuals using the multiplex PCR technique. Of these, 135 showed no change, 18 showed partial deletions and 12 showed total deletions of the *hprt* gene.

Examples of multiplex *hprt* PCR patterns are shown in Figure 3. GM131 is an apparently normal lymphoblastoid cell line with an intact *hprt* gene and exhibits the wild-type

Table 1. Analysis of *hprt* mutant frequency.

Donor code	Age (years)	Sex	Smoking status[a]	Cloning efficiency, %	Mutant frequency, × 10^{-6}	No. of cells plated, × 10^6	Total no. of mutants[b]
A	28	M	NS	29	8	10	23
B	28	M	NS	29	2	10	6
C	31	M	LS	44	5	20	41
D	31	M	LS	8	10	20	15
E	26	M	NS	18	3	9	4
F	21	M	LS	22	8	16	27
G	24	F	H	43	6	10	24
H	28	F	LS	40	5	13	28
I	21	M	LS	20	15	13	39
J	22	M	LS	41	4	10	16
K	24	F	H	53	4	12	26
L	28	F	LS	44	9	12	42
M	21	M	LS	51	6	12	36
N	26	M	NS	27	12	58	167
O	70	F	NS	33	16	4	22
P	67	M	NS	46	10	5	23
Q	70	M	NS	54	27	8.5	95
R	65	M	NS	51	13	11.5	75
S	70	F	NS	12	18	1.7	4

[a]Smoking status: NS, nonsmoker; LS, light smoker (1–15 cigarettes/day); h, heavy smoker (>15 cigarettes/day).
[b]Total number of *hprt* mutants scored from the sample analyzed.

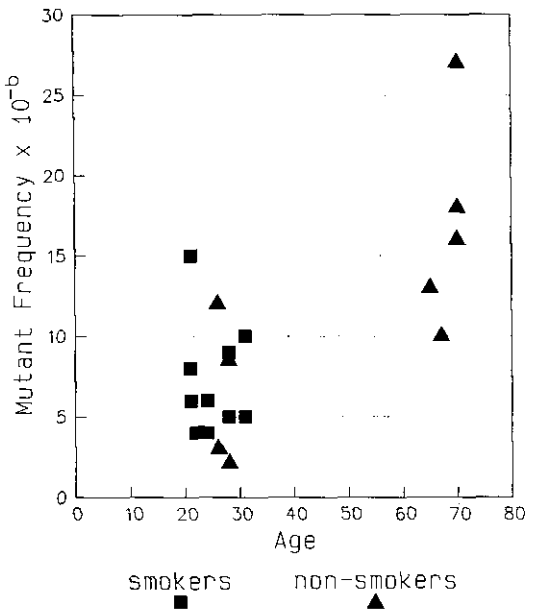

FIGURE 2. The effect of age and smoking on the quantitated mutant frequency.

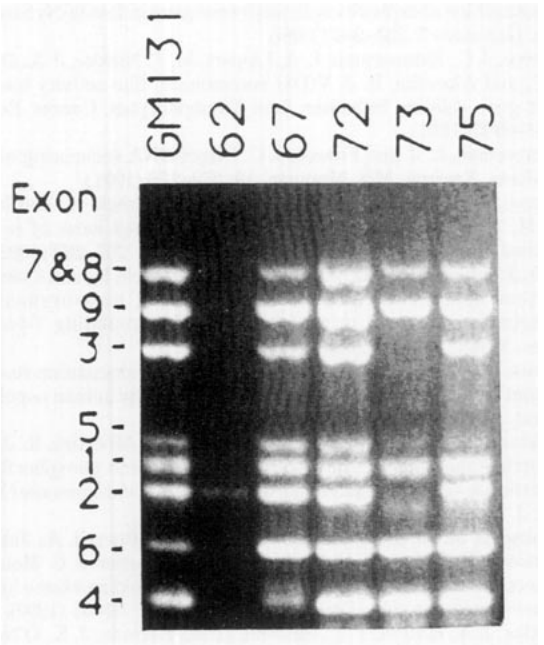

FIGURE 3. Multiplex *hprt* PCR analysis of HPRT-deficient human T-lymphocyte clones. PCR products are displayed on a 1.4% agarose gel. Mutants 62, 67, 72, 73, and 75 are from individual Q (Table 1). GM131 is a lymphoblastoid line with a normal *hprt* gene. DNA from this line displays the wild-type pattern.

pattern, as do mutants 72 and 75. Mutant 62 does not give a signal and was subsequently found to be due to a total *hprt* gene deletion by the K-*ras* + *hprt* exon 6 duplex PCR assay. Mutant 67 shows a deletion of exon 4, and other exons appear normal. We have found that exon 4 is the exon most frequently involved in small deletions. Mutant 73 shows loss of exons 2 and 3. We have recently shown that illegitimate activity of V(D)J recombinase causes deletion of *hprt* exons 2 and 3 (*10*). This mutant is, therefore, a candidate for this type of mutation.

In an attempt to obtain more mutants with total *hprt* deletions, we performed several *in vitro* treatments with bleomycin. After several unsuccessful attempts using the standard method to grow mutants to sufficient numbers for molecular analysis, we used the *in situ* method developed by O'Neill (*7*). Perhaps because the individual mutants get a "head start" of several cell divisions in the wells during the expression phase before the addition of the selective agent, we were more successful in culturing mutants using this technique. Although the bleomycin-induced mutant frequency was low in these experiments and the number of mutants available for study likewise low, we were successful in finding 4-total deletion and 1 partial-*hprt*-deletion mutants out of 17 mutants studied (Table 2). Of these mutants, 2 (one partial and one total *hprt* gene deletion) came from an untreated culture.

Because the TK6 feeder-cell line contains a nearly intact *hprt* gene, this DNA can serve as a substrate in the PCR reactions and give rise to faint signals. (This can also be a problem in Southern blot analyses). These signals can make interpretation of the PCR gels difficult and may require additional experiments to resolve. To avoid this problem, we sought a feeder-cell line that was missing the *hprt* gene and a large amount of flanking DNA. X3C (*4*) was found by multiplex *hprt* PCR and K-*ras*/exon 6 duplex PCR to be missing the entire *hprt* gene. By coamplifying a small region, about 350 kb 5′ of the *hprt* gene with the unlinked K-*ras* gene segment, the deletion was found to include at least 350 kb upstream of the gene. Similarly, duplex PCR with K-*ras* and a segment approximately 300 kb 3′ of *hprt* showed the deletion extended at least that far downstream of *hprt*. Thus, cell line X3C contains a deletion of at least 700 kb around the *hprt* gene. This cell line has proven comparable to TK6 in supporting the growth of T-lymphocytes in the *hprt* T-cell cloning assay (data not shown) and has greatly simplified molecular analysis.

Conclusions

Significant progress has been made in understanding *in vitro* gene mutation assays. Quantitative comparisons between autosomal markers and X-linked markers have demonstrated that autosomal markers, like the *tk* gene, can detect the clastogenicity of chemicals, whereas X-linked markers, like *hprt*, severely underestimate the clastogenicity of test chemicals (*3*). There have been two hypotheses to explain these differences: First, mutants with deletions extending outside of the *hprt* locus are not viable, but deletions extending outside of the *tk* locus are viable. Second, a substantial number of *tk* mutants result from mitotic recombination, a mechanism not available to hemizygous loci. Our current studies and those by Nicklas et al. (*15*) show that large deletions around *hprt* are

Table 2. Mutant frequency and molecular analysis of *hprt* mutants from the *in vitro* treatment of lymphocytes with bleomycin using the *in situ* technique.

Bleomycin, μg/mL	No change[a]	Partial deletion	Total deletion	No. of mutants[b]	Unselected cloning efficiency	Mutant frequency, $\times 10^{-6}$
0	6	0	0	6	20	8
100	1	0	2	3	9	71
0	2	1	1	4	19	7
100	3	0	1	4	10	72

[a]Number of mutants showing no change of band position using the multiplex *hprt* PCR anaylsis.
[b]Total number of mutants analyzed.

tolerated. This suggests that the difference is not due to an inability of the *hprt* locus to sustain large deletions. Furthermore, our earlier investigations of *tk* mechanisms of mutagenesis (*16*) suggest that a significant number of *tk* mutants result from homologous mitotic recombination.

In the present studies, we have demonstrated our ability to produce mutant frequency data similar to that published by other laboratories. We have also begun to evaluate the range and types of genetic damage detected by the human T-lymphocyte *hprt* assay. Based on our data and that of other laboratories, we know that the spectrum of genetic damage detected includes point mutations (*17,18*) and deletions ranging from a few base pairs, one or more exons, the entire *hprt* gene and extensive numbers of base pairs outside of the *hprt* gene. We are currently working to define the size of the maximum-tolerated deletion around the *hprt* gene as well as to determine the mechanism(s) of deletion formation.

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